

Helicobacter anseris sp. nov. and *Helicobacter brantae* sp. nov., Isolated from Feces of Resident Canada Geese in the Greater Boston Area

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Numbers of nonmigratory Canada geese have increased substantially in the past decade, and they have become a nuisance in some urban areas. Because of their close contact with humans in parks and areas adjacent to surface waterways, contact with their feces poses a zoonotic risk. A total of 97 geese from 10 separate geographic locales in the greater Boston area had their feces sampled for detection of *Helicobacter* spp. Identification of *Helicobacter* spp. based on 16S rRNA genus-specific helicobacter primers was noted in 39 of 97 (40.2%) DNA fecal extracts. Twenty-seven (27.8%) of these geese had helicobacters isolated from their feces. A urease-positive novel species, *Helicobacter anseris*, based on phenotypic, biochemical, and 16S rRNA analyses, was isolated from 20 geese from seven different flocks. A second, novel, urease-negative *Helicobacter* sp., *H. brantae*, was identified in seven geese. Four geese had both novel *Helicobacter* spp. cultured from their feces. Whether these two novel helicobacters pose a zoonotic risk, similar to other enteric helicobacters (e.g., *H. canadensis*, previously isolated from diarrheic and bacteremic humans and from geese in Europe), will require further studies.

The genus *Helicobacter* comprises a group of gram-negative, microaerophilic, spiral to curve-shaped bacteria originally isolated from the stomachs of humans and other mammals (11). The type species of the genus, *Helicobacter pylori*, causes chronic gastritis and peptic ulcer disease in humans and has more recently been linked to the development of gastric adenocarcinoma and gastric mucosa-associated lymphoma (3, 4, 7). Since *H. pylori* was first isolated in 1982, an additional 26 formally named *Helicobacter* spp. have been characterized. These have been cultured from a variety of hosts, most commonly the gastrointestinal tracts of mammals (11).

Wild birds have been recognized for decades as reservoirs of *Campylobacter* spp. (8, 17, 22). However, it was not until *H. pametensis* was isolated from wild birds and named in 1994 that avian species were recognized as reservoirs of enteric helicobacters (6, 19). *Helicobacter pametensis* was also isolated from the feces of a pig. Other helicobacters not formally named but referred to as “helicobacter bird B” and “helicobacter bird C” were also cultured from the feces of wild birds living on the coast of Massachusetts (6). Soon thereafter, *H. pullorum* was isolated from the intestinal tracts of chickens, diseased chicken livers, and diarrheic humans (20). Most recently, *H. canadensis*, first isolated from diarrheic and bacteremic patients, was cultured from the feces of Barnacle geese (*Branta leucopsis*) and Canada geese (*Branta canadensis*) on the Atlantic coast of Europe (12, 21–23).

Because *H. canadensis* was originally identified in humans and subsequently found in geese, we wanted to ascertain whether this helicobacter would also be isolated from Canada

geese which frequent public waterways, parks, and golf courses (2, 8, 9). Their feces heavily contaminate those areas and could serve as a zoonotic source of enteric helicobacters, including *H. canadensis*. This study describes the isolation and characterization by phenotypic, biochemical, 16S rRNA, and 23S rRNA analyses of two novel helicobacters, *Helicobacter anseris* and *H. brantae*, from the feces of Canada geese in the greater Boston, Massachusetts, area.

MATERIALS AND METHODS

Animals. During the summer of 2004, 10 different flocks, totaling 97 geese, from 10 different geographic areas within a 10-mile radius of Boston were sampled, and their feces were cultured and analyzed by genus-specific PCR for *Helicobacter* spp. Individual geese were identified by two of the authors by observing the flocks for fresh fecal samples, which were collected in sterile vials containing 20% glycerol brucella broth. These were stored on ice and transported to the laboratory. The vials were then frozen at -70°C prior to processing, and within a 6-month period all samples were cultured.

Bacterial isolation and biochemical characterization. Samples from each fecal sample were homogenized in 1.0 ml of phosphate-buffered saline. Each slurry was gently passed through a 0.45- μm -pore-size filter onto a Trypticase soy agar (TSA) plate (with 5% sheep blood) (Remel, Lenexa, Kans.). The homogenate was streaked directly onto TSA plates containing either trimethoprim, vancomycin, and polymyxin B or cefoperazone, vancomycin, and amphotericin B (Remel). In addition, a selective antibiotic medium was prepared with the following agents: blood agar base (Oxoid; Remel), 5% horse blood (Remel), 50 μg of amphotericin B/ml, 100 μg of vancomycin/ml, 3.3 μg of polymyxin B/ml, 200 μg of bacitracin/ml, and 10.7 μg of nalidixic acid/ml (all from Sigma Chemical Co., St. Louis, Mo.). The cultures were then incubated at 37°C under microaerobic conditions in vented jars containing N_2 , H_2 , and CO_2 (80:10:10). A detailed biochemical characterization analysis was performed on a minimum of seven isolates of each novel *Helicobacter* sp., as previously described by Fox et al. (14).

Electron microscopy. Two isolates, one representing *H. anseris* and the other representing *H. brantae*, were examined by electron microscopy. Cells grown on blood agar for 48 h were gently suspended in 10 mM Tris-HCl buffer (pH 7.4) at a concentration of about 10^8 cells per ml. Samples were negatively stained with 1% (wt/vol) phosphotungstic acid (pH 6.5) for 20 to 30 s. Specimens were

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TABLE 1. PCR results for goose flocks

Flock no.	No. of geese	No. of <i>Helicobacter</i> -positive isolates by fecal PCR	No. of isolates		Matching 16S rRNA sequence	
			<i>H. anseris</i>	<i>H. brantae</i>	<i>H. anseris</i>	<i>H. brantae</i>
1	10	2	0	0		
2	10	4	0	0		
3	10	5	3	1	04-9362 ^T , 04-6161, 04-6162	
4	10	2	1	1		04-9366 ^T
5	10	3	3	0	04-7461, 04-7462	
6	10	5	4	0	04-7465	
7 ^a	10	4	3	2		04-7467
8	10	7	4	2	04-9367	
9	7	4	0	1		
10	10	2	2	0	04-9365	04-9364
Total (%)	97	39 (32)	27 (27.8) (both <i>H. anseris</i> and <i>H. brantae</i>)		8	3

^a Includes Canada geese and domesticated white geese hybrids.

examined with a JEOL model JEM-1200EX transmission electron microscope operating at 100 kV.

Genomic DNA extraction for rRNA gene sequencing. Bacteria isolated from the feces of 11 geese were cultured on blood agar plates, and cells were harvested and washed once with 1 ml of phosphate-buffered saline. A High Pure PCR template preparation kit was used for DNA extraction (Roche Molecular Biochemicals, Indianapolis, Ind.).

16S rRNA gene sequencing. The sequences of the 16S rRNA genes of 11 isolates of *Helicobacter* spp. were determined (Table 1). Amplification of 16S rRNA genes, sequencing, and 16S rRNA data analysis were performed as described previously by Fox et al. (13). Briefly, primers C70 and B37 were used to amplify the 16S rRNA genes. The amplicons were purified and directly sequenced by using a TAQuence cycle sequencing kit (U.S. Biochemicals, Cleveland, Ohio). The 16S rRNA gene sequences were entered into RNA, a program for analysis of 16S rRNA data, and were aligned as described previously (16). Similarity matrices were constructed from the aligned sequences by using only those base positions for which 90% of the strains had data and were corrected for multiple base changes by the method of Jukes and Cantor (15). Phylogenetic trees were constructed by the neighbor-joining method (18).

23S rRNA gene sequences. Complete 23S rRNA gene sequences (>2,700 bases) were determined for one strain of *Helicobacter anseris* and one strain of *H. brantae*. These strains were sequenced on both strands, using 16 to 20 primers, as previously described (5).

***Helicobacter* sp.-specific PCR amplification.** Primers specific for amplifying helicobacter 16S rRNA genes were used to determine the presence of helicobacters in fecal samples, as previously described (13). This set of primers produces an amplicon of 1.2 kb. Briefly, 20 µl of the DNA preparation was added to 100 µl of a reaction mixture containing 1× *Taq* polymerase buffer (supplied by the manufacturer but supplemented with 1 M MgCl₂ to a final concentration of 2.25 mM), 0.5 µM (each) primers, a 200 µM concentration of each deoxynucleotide, and 200 µg of bovine serum albumin per ml. Samples were heated at 94°C for 4 min, briefly centrifuged, and cooled to 58°C. At this time, 2.5 U of *Taq* polymerase (Roche Molecular Biochemicals) and 1.0 U of polymerase enhancer (Perfect Match; Stratagene, La Jolla, Calif.) were added, and then the samples were overlaid with 100 µl of mineral oil. For amplification of the 1.2-kb fragment, the following conditions were used: 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 2 min, and elongation at 72°C for 3 min, followed by an elongation step of 8 min at 72°C. Fifteen microliters of the sample was then electrophoresed through a 1% agarose gel, followed by ethidium bromide staining, and samples were visualized by UV illumination.

Nucleotide sequence accession numbers. The GenBank accession numbers for sequences determined in this study are as follows: *H. anseris* 16S rRNA, DQ415545; *H. anseris* 23S rRNA, DQ418749; *H. brantae* 16S rRNA, DQ415546; and *H. brantae* 23S rRNA, DQ418750.

RESULTS

Prevalence of *Helicobacter* spp. by PCR and culture. All 10 flocks surveyed in the greater Boston area had helicobacters

identified in their feces by *Helicobacter* genus-specific PCR (Table 1). *Helicobacter* spp. were detected in 39/97 (40.2%) fecal samples by PCR. For 27 (27.8%) of the fecal samples, helicobacters were isolated by culture. These helicobacter isolates were from eight flocks and were confirmed by *Helicobacter* genus-specific PCR.

Biochemical characterization. The isolates clustered into two novel phenotypic groups. The first group was urease, catalase, and oxidase positive, hydrolyzed indoxyl acetate, and reduced nitrate but did not have γ-glutamyl transpeptidase activity. The strains grew at 42°C in the presence of 1% glycine and were susceptible to nalidixic acid but resistant to cephalothin. For this group of novel helicobacter isolates, we propose the name *Helicobacter anseris*. The second novel group of isolates differed from the first by being urease negative, showed intermediate susceptibility to cephalothin, and growing weakly in the presence of 1% glycine (Table 2). For the second group of novel isolates, we propose the name *H. brantae*. *H. brantae*, which is closely related to *H. cholecystus* by 16S rRNA analysis (98.5% similarity), can be distinguished from *H. cholecystus* biochemically by being unable to reduce nitrate to nitrite and having indoxyl acetate hydrolysis activity.

Electron microscopy. A representative of *Helicobacter anseris*, MIT 04-9362, was a slightly curved to rod-shaped bacterium measuring 0.5 by 1.8 µm with single sheathed flagella located subterminally at each end (Fig. 1).

A representative of *H. brantae*, MIT 04-9366, was a slightly curved rod measuring 0.5 by 1.5 µm with single sheathed flagella positioned at the subterminal ends (Fig. 2).

16S rRNA and 23S rRNA analysis. Eleven goose helicobacter isolates were analyzed by 16S rRNA gene sequencing. The eight urease-positive *H. anseris* isolates had identical sequences and clustered in the 16S rRNA phylogenetic tree near helicobacter bird C (Fig. 3). The three urease-negative *H. brantae* isolates had identical sequences and clustered in the 16S rRNA tree near *H. cholecystus*. 23S rRNA sequences were determined for one isolate from each novel group. A phylogenetic tree based on the 23S rRNA sequence analysis is shown in Fig. 4. The *H. anseris* isolate was 97.9% identical to the sequence for helicobacter bird B and 96.4% identical to *H.*

TABLE 2. Biochemical characteristics of *Helicobacter* spp. isolated from birds^a

Species	Catalase production	Urease activity	Oxidase production	Nitrate reduction	Alkaline phosphatase hydrolysis	Indoxyl acetate hydrolysis	γ -Glutamyl transpeptidase activity	Growth		Susceptibility	
								At 42°C	With 1% glycine	Nalidixic acid ^b	Cephalothin ^b
<i>H. anseris</i>	– (12/12)	+	+	– (12/12)	– (12/12)	+	– (0/12)	+	Weak + (12/12)	S (12/12)	R (12/12)
<i>H. brantae</i>	– (7/7)	– (0/7)	+	– (7/7)	– (7/7)	+	– (0/7)	+	Weak + (7/7)	S (7/7)	I (7/7)
Helicobacter bird B	+	+	+	+	+	+	–	+	+	S	R
Helicobacter bird C	+	–	+	+	+	–	–	+	+	S	S
<i>H. pametensis</i>	+	–	+	+	–	+	–	+	+	R	R
<i>H. canadensis</i>	+	–	+	+	–	–	ND	+	–	R	S
<i>H. pullorum</i>	+	–	+	+	–	–	ND	+	–	R	S

^a Values in parentheses represent numbers of isolates/total number of isolates.

^b Thirty-microgram disk. S, sensitive; R, resistant; I, intermediate.

brantae, whereas *H. brantae* was 98.5% identical to *H. cholecystus*.

DISCUSSION

The identification of *H. anseris* and *H. brantae* as novel species is based on a polyphasic analysis of both phenotypic and genotypic traits. These novel species differ from other *Helicobacter* spp. by two to five major traits (Table 2) (10). Interestingly, the subterminal placement of the flagella near the ends of the bacteria for both *H. anseris* and *H. brantae* is rarely seen in *Helicobacter* spp. but is also a feature of *H. pametensis*, another bird helicobacter (6). It is noteworthy that 16S rRNA sequence analysis suggests that *H. anseris* and helicobacter bird C are the same species (99.5% identity) but 23S rRNA sequence analysis suggests that they are distinct species (96.2% identity). As we previously reported, 16S rRNA and 23S rRNA sequence inferences can be discordant, and the 23S rRNA information appears to be more congruent with phenotypic information (5).

These novel helicobacters were isolated from resident geese inhabiting parks and public areas routinely frequented by local inhabitants, including children. Geese excreta in these public areas have increasingly raised the public's annoyance level from both a sanitation perspective and, more importantly, their possibility as a source of infections to humans via zoonotically

transmitted microorganisms. It is suspected, though not definitely proven, that another enteric helicobacter colonizing chickens, i.e., *H. pullorum*, is directly transmitted via fecal contamination to humans. This organism has been associated with diarrhea and bacteremia in humans (20). Recently, in a large survey of 600 humans, *H. pullorum* was identified by specific PCR in 4.1% of feces from both diarrheic and nondiarrheic humans (1). In addition to diarrheal disease in humans, *H. pullorum* has also been isolated from livers of chickens with hepatitis (20). Other enterohepatic helicobacters, including *H. cinaedi*, *H. hepatitis*, *H. bilis*, and *H. canis*, have been isolated from diseased livers of several mammalian species (11). Furthermore, *H. canadensis*, originally misclassified as *H. pullorum*, is associated with diarrhea and bacteremia in humans and was recently isolated from the feces of geese inhabiting coastal areas in Europe (12, 21, 23). The isolation of novel helicobacters from the feces of geese collected from the grounds of public parks may pose a similar zoonotic risk, as well as possibly infecting other species of birds and mammals. The pathogenic potentials of *Helicobacter anseris* and *H. brantae* are currently unknown.

Other related microaerophiles, such as *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter lari* colonize

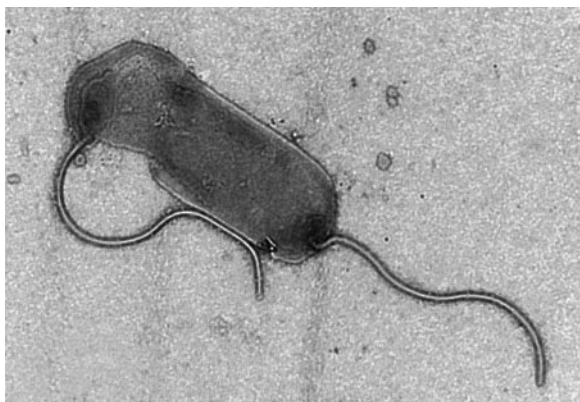


FIG. 1. *H. anseris*, a slightly curved rod with subterminal sheathed flagella.

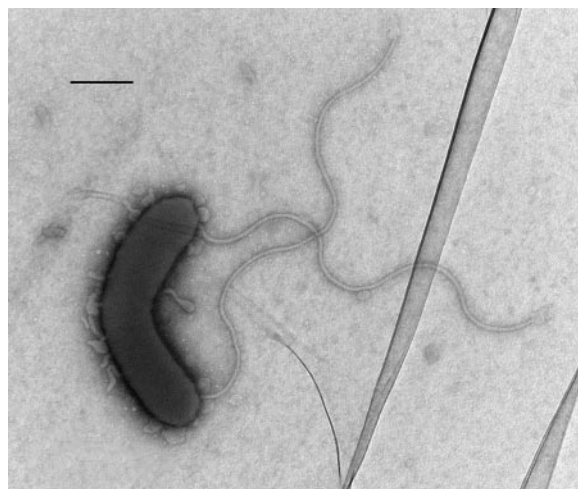


FIG. 2. *H. brantae*, a curved rod with subterminal sheathed flagella. Bar = 0.5 μ m.

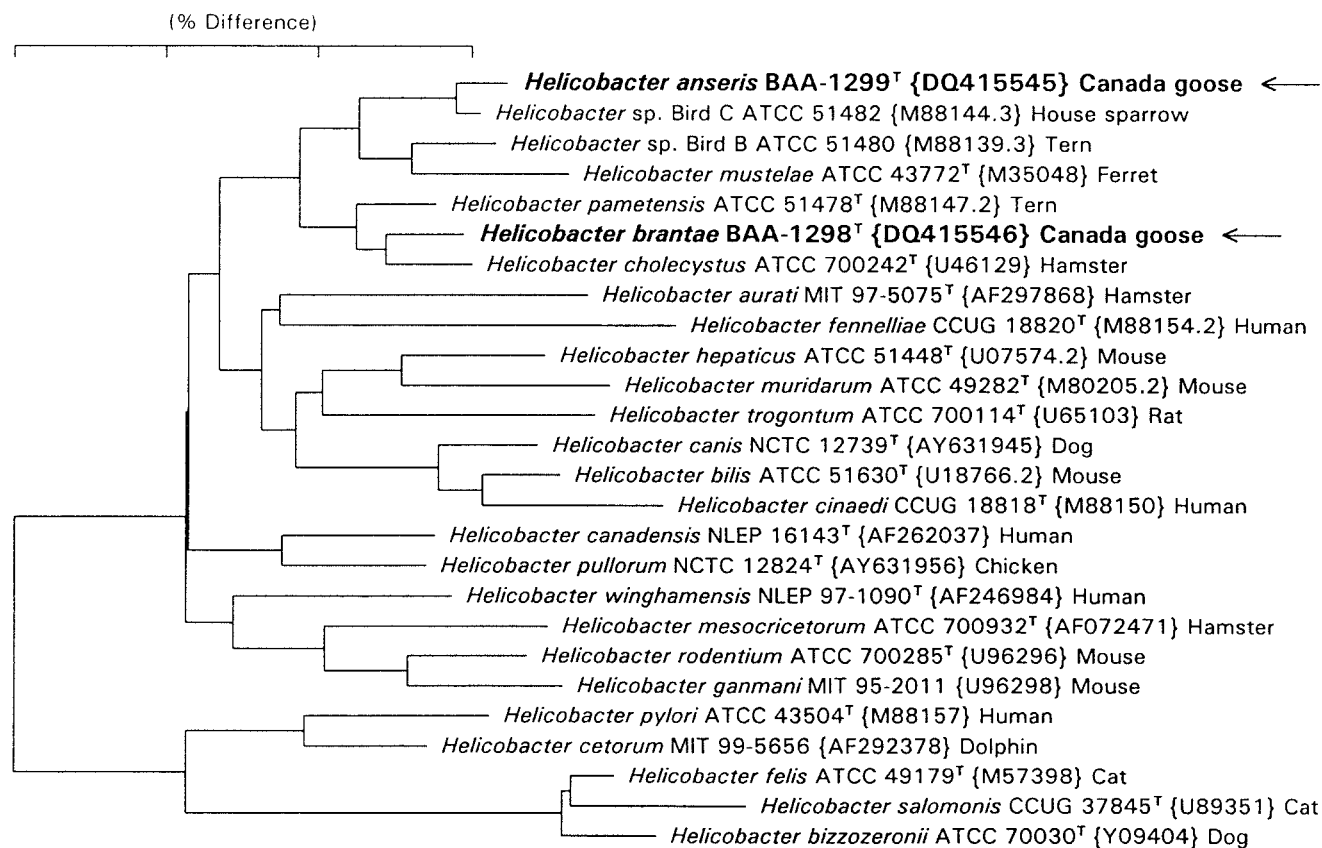


FIG. 3. Phylogenetic tree constructed on the basis of 16S rRNA sequence similarity values. The scale bar is equal to a 3% difference in nucleotide sequences, as determined by measuring the lengths of the horizontal lines connecting two species.

a variety of migratory birds (22). It is assumed, but not proven, that these birds are also a source of campylobacter infection in humans. However, serotypes of wild bird isolates differed from those of human and chicken isolates, suggesting that wild birds may be less important than poultry in zoonotic spread of *Campylobacter* spp. (17). Similar molecular typing of *Helicobacter* spp. isolated from birds and humans is required to assess their zoonotic potential. As with campylobacter infections in birds, the epidemiology of *Helicobacter* spp., including *H. anseris* and *H. brantae*, in

birds will require a thorough understanding of feeding habits, migrating patterns, habitat preferences, and life spans of individual bird species. It is clear, however, that the increasing presence of Canada geese and their proximity to urban locales on a year-round basis increase the likelihood that these birds could be a source of zoonotic transmission through direct fecal contamination or via fecal contamination of water. This possibility heightens the need to further study and identify intestinal microflora of Canada geese for assessment of zoonotic potential.

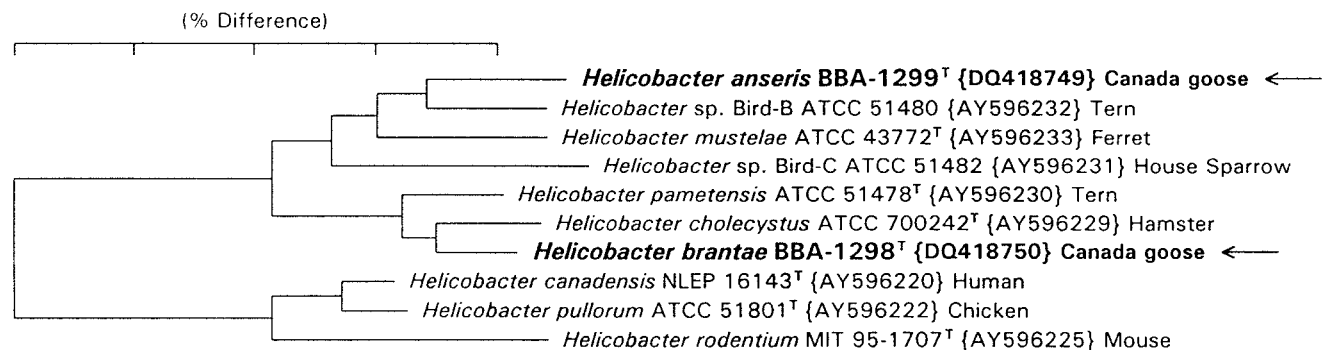


FIG. 4. Phylogenetic tree constructed on the basis of 23S rRNA sequence similarity values. The scale bar is equal to a 4% difference in nucleotide sequences, as determined by measuring the lengths of the horizontal lines connecting two species.

Description of *H. anseris* sp. nov. (anseris. L. gen. masc./fem. n. anseris, of the goose). Cells are slender and slightly curved to rod shaped (0.5 by 1.8 μ m). The bacterium is gram negative and nonsporulating; it is motile by virtue of possessing single sheathed flagella located subterminally at each end. Cultures grown on solid agar media appear as a spreading layer. Organisms grow under microaerobic conditions but not aerobically or anaerobically. Growth occurs at 37°C and 42°C. The bacterium is oxidase, catalase, and urease positive but alkaline phosphatase and γ -glutamyl transpeptidase negative. The organism hydrolyzes indoxyl acetate and grows in 1% glycine but does not reduce nitrate to nitrite. Cells are sensitive to nalidixic acid but resistant to cephalothin. Bacteria have been isolated from the feces of Canada geese. The type strain is MIT 04-9362 (ATCC BAA-1299). The 16S rRNA sequence accession number for the type strain is DQ415545, and that for the 23S rRNA is DQ418749.

Description of *H. brantae* sp. nov. (bran'tae. N.L. gen. fem. n. Branta, the zoological genus name of Canada geese). Cells are slender and slightly curved to rod shaped (0.5 by 1.8 μ m). The bacterium is gram negative and nonsporulating; it is motile by virtue of possessing single sheathed flagella located subterminally at each end. Cultures grown on solid agar media appear as a spreading layer. Organisms grow under microaerobic conditions but not aerobically or anaerobically. Growth occurs at 37°C and 42°C. The bacterium is oxidase and catalase positive but urease, alkaline phosphatase, and γ -glutamyl transpeptidase negative. The organism hydrolyzes indoxyl acetate and grows in 1% glycine but does not reduce nitrate to nitrite. Cells are sensitive to nalidixic acid but resistant to cephalothin. Bacteria have been isolated from the feces of Canada geese. The type strain is MIT 04-9366 (ATCC BAA-1298). The 16S rRNA sequence accession number for the type strain is DQ415546, and that for the 23S rRNA is DQ418750.

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S.H. and M.T. identified and collected samples from individual geese.

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